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Dynamic Effects of Free Chlorine Concentration, Organic Load, and Exposure Time on the Inactivation of *Salmonella, Escherichia coli* O157:H7, and Non-O157 Shiga Toxin–Producing *E. coli*†

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ABSTRACT

This study evaluated the dynamic effects of free-chlorine (FC) concentration, contact time, and organic load on the inactivation of *Salmonella, Escherichia coli* O157:H7, and non-O157 Shiga toxin–producing *E. coli* (STEC) in suspension. Bacterial cells from four strains each of *Salmonella, E. coli* O157:H7, and non-O157 STEC were inoculated separately or as a multistrain cocktail into solutions with varying FC concentrations. Lettuce or tomato extract was used to simulate the organic matter present during commercial fresh and fresh-cut produce wash operations. After exposure to FC for various lengths of time, the bacterial survival and water-quality changes were determined. In the absence of organic matter in a wash solution, pathogen inactivation is primarily a function of initial FC concentration ($P < 0.0001$), exposure time ($P < 0.0001$), and pathogen strains ($P < 0.0001$). In general, an over 4.5-log CFU/ml pathogen reduction was found after exposure to >0.5 mg/liter FC for over 30 s, or to >1.0 mg/liter FC for over 5 s. When the combination of FC concentration and contact time were less than or equal to the above conditions, survival of pathogens was strain dependant and ranked as: *Salmonella* > *E. coli* O157:H7 > non-O157 STEC. When organic matter was present in the wash solution, pathogen inactivation efficacy was specifically dependent on the residual FC concentration, which directly relates to both the initial FC concentration and the organic load. Prevention of pathogen survival in chlorinated produce wash solutions can be achieved by maintaining sufficient FC concentration and reducing the accumulation of organic matter.

Foodborne illness outbreaks associated with the consumption of contaminated fresh and fresh-cut produce have been well documented in the past 20 years. Examples include tomatoes contaminated with *Salmonella (12),* and lettuce contaminated with *Escherichia coli* O157:H7 *(22)* or non-O157 Shiga toxin–producing *E. coli* (STEC) *(18)*. Washing is one of the most critical processing steps during fresh produce preparation to inactivate pathogens and improve product quality, safety, and shelf life *(10)*. However, washing also has the potential to cause pathogen cross-contamination in the absence of effective disinfectants, especially when water is reused and recirculated *(10, 16, 17).* Although several commercially available chemical sanitizers such as chlorine dioxide, ozone, acidified sodium chlorite, and peroxyacetic acid are approved for use in fresh-cut washes *(24),* chlorine continues to be the most commonly used sanitizer in the fresh produce industry *(5, 13)*. This prevalence of commercial chlorine usage is attributable to its established ability to kill pathogens in solution, minimal impact on product quality, and low cost *(2, 6, 11, 14)*.

In aqueous solutions near neutral pH, free chlorine (FC) is predominately present as hypochlorous acid (HOCl), the most effective form of chlorine for disinfection *(1)*. Alkaline pH favors the conversion to the less efficacious hypochlorite ion, OCl^-, while acidic pH favors the formation of gaseous chlorine Cl_2, which tends to be released from solution as off-gas in substantial amounts when the pH is below 4.0 *(25)*. Several studies have shown that the pathogen inactivation efficacy of chlorine solutions is highly dependent on the concentration of hypochlorous acid and the exposure time *(20, 27)*. However, many of those studies were conducted without considering the effect of organic matter in wash solutions.

Chlorine is a strong oxidant and reacts with organic matter quickly, leading to rapid decline in its concentration and sanitation efficacy. This is especially problematic for fresh-cut produce wash, as wash solutions contain a large amount of organic matter from cut-produce tissue exudates, soil, and other debris. Chlorine needs to be frequently added to the wash system in order to compensate for the loss of FC. However, there is a lack of scientific information pertaining to the timing and the ability of the intervention to maintain the sanitation strength of the wash solution without incurring the detrimental side effects of overdosing.

In recent studies, we evaluated the minimum chlorine concentration required to prevent cross-contamination for fresh-cut produce wash. It was determined that although

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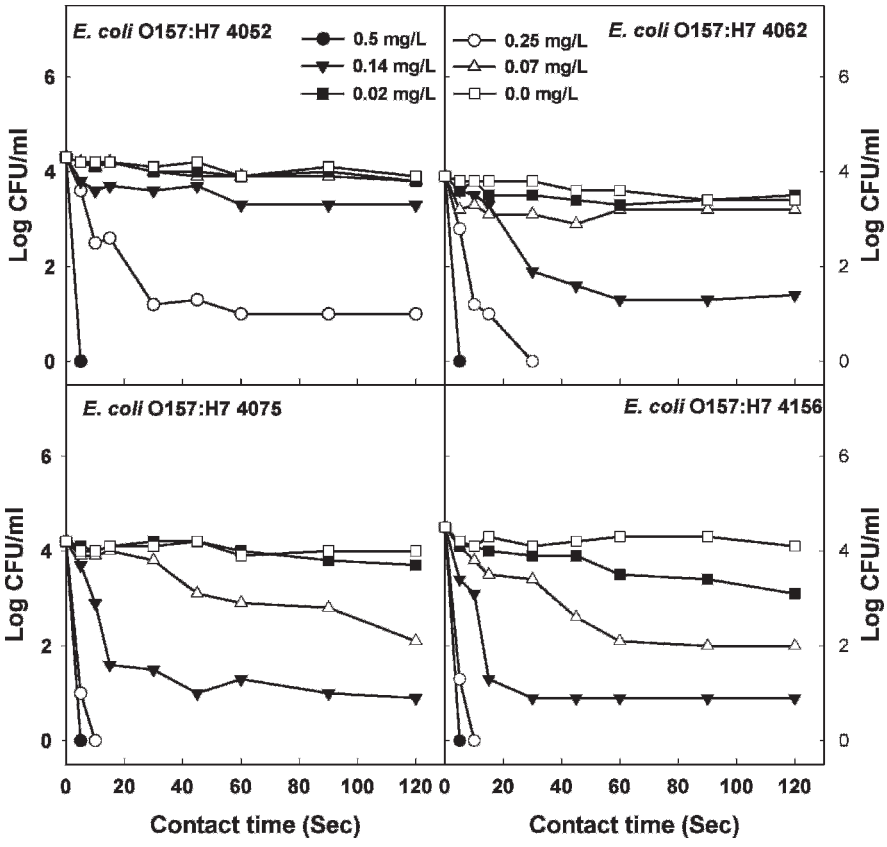

FIGURE 1. *The effect of FC concentration and exposure time on the inactivation of* Escherichia coli *O157:H7 strains 4052, 4062, 4075, and 4156 in suspension.*

1 mg/liter FC was sufficient to prevent pathogen survival in the wash solution after 30 s of exposure, 10 mg/liter FC was needed to prevent pathogen cross-contamination during produce wash *(17)*. This discrepancy was accounted for by the hypotheses that cross-contamination can be mediated by leaf contact, and that pathogens washed off the contaminated produce might be able to reattach to the clean produce before they are killed in the solution. The earlier version of

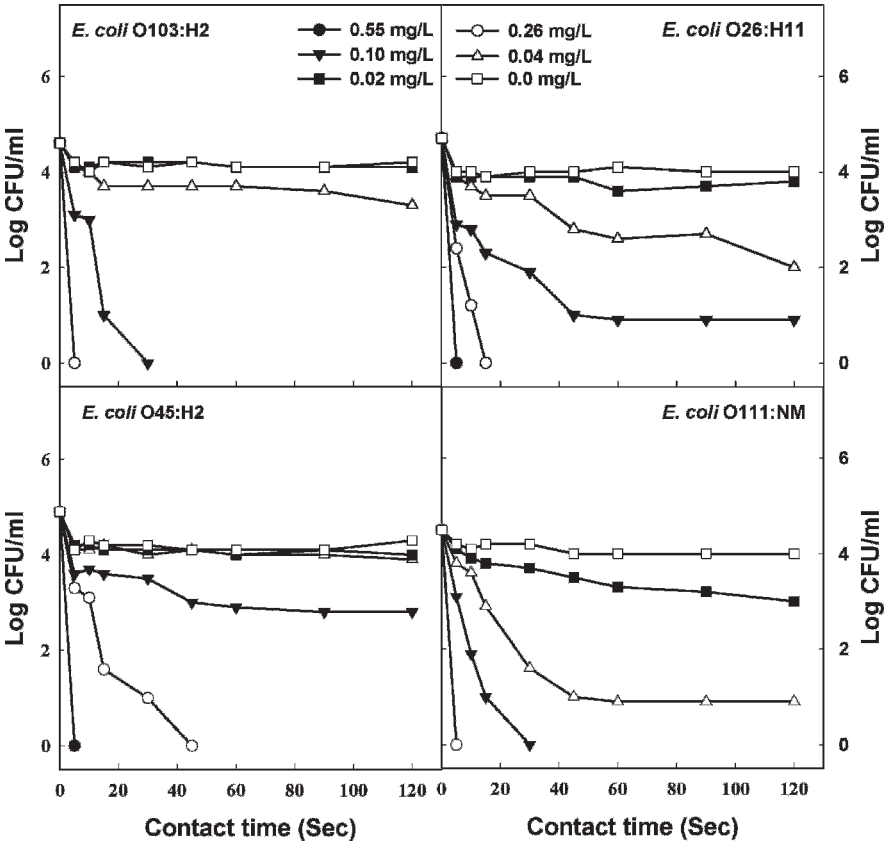

FIGURE 2. *The effect of FC concentration and exposure time on the inactivation of non-O157 STEC strains* Escherichia coli *O103:H2, O26:H11, O45:H2, and O111:NM in suspension.*

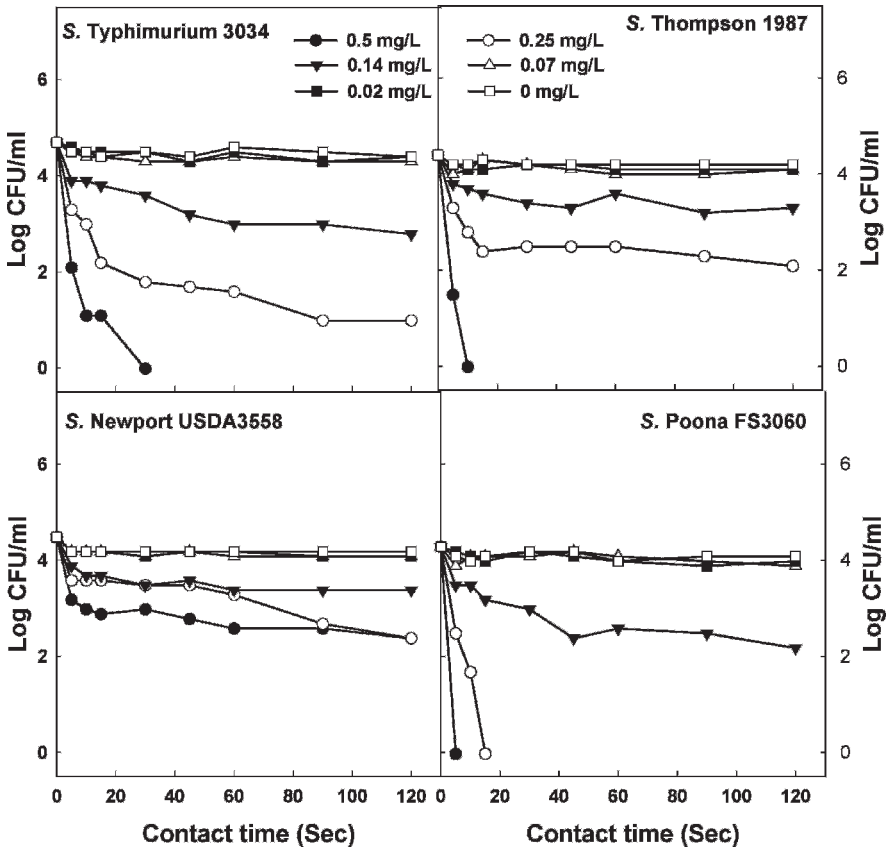

FIGURE 3. *The effect of FC concentration and exposure time on the inactivation of* Salmonella *enterica Typhimurium, Thompson, Newport, and Poona in suspension.*

Florida Department of Agriculture's *Tomato Good Agricultural Practices* required a minimum of 150 mg/liter FC at pH 6.5 to 7.5 in dump tank and flume water to prevent the survival and potential internalization of human pathogens in tomatoes *(3)*. In the absence of scientific studies substantiating the need for this high chlorine concentration and the uncertainty surrounding the appropriate concentration of FC for pathogen control in dump tanks and flumes, the option to maintain an oxidation-reduction potential (ORP) value of 650 mV instead of monitoring for chlorine was later included *(4)*. However, a number of recent reports also questioned the reliability of ORP measurement as a proxy for the disinfection potential of water in a postharvest wash system, and the linearity of correlation between ORP reading and effective sanitizer strength *(21, 23)*. The importance of the impact of organic load on the efficacy of wash-water sanitizers is recognized. However, information on the effect of organic materials typically encountered during tomato and leafy green wash operations on the degradation of sanitizer concentration and efficacy to inactivate pathogens is lacking. In this study, we report our findings on the inactivation of *Salmonella, E. coli* O157:H7, and non-O157 STEC in chlorinated wash solutions across a range of FC concentrations, contact times, and organic loads. Results should provide useful information for the industry to develop practical procedures to validate the efficacy of wash-water or flume-water microbial control systems during produce washing, a top research priority identified by the Center for Produce Safety *(7)*.

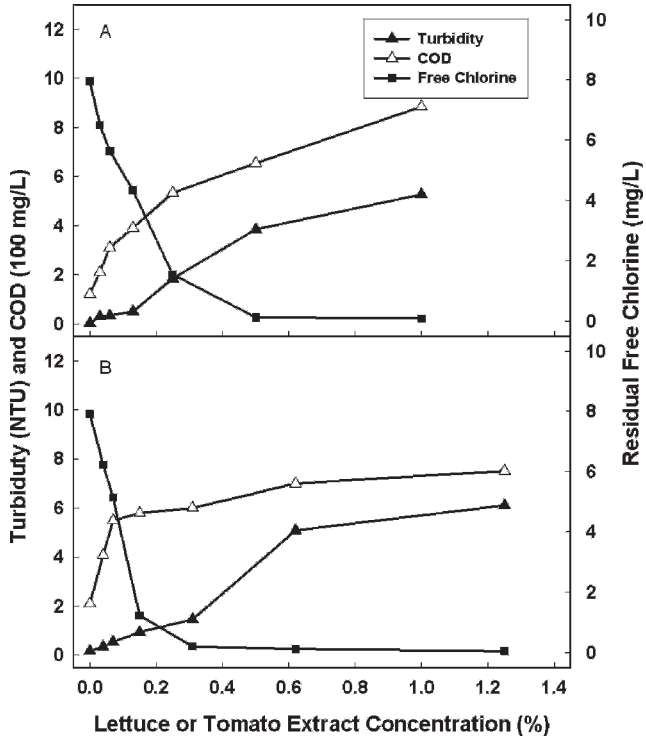

FIGURE 4. *Changes in solution COD and residual FC concentration as impacted by the organic load simulated with lettuce extract (A) or tomato extract (B).*

TABLE 1. *Residual FC concentration as impacted by the amount of LE–organic load and the initial FC concentration*

		Residual FC concn (mg/liter) with initial concn (mg/liter) of:				
LE (%)	COD (mg/liter)	2.19 ± 0.16	1.09 ± 0.06	0.53 ± 0.05	0.27 ± 0.03	0.12 ± 0.01
0	112	1.93 ± 0.19	0.85 ± 0.09	0.48 ± 0.06	0.22 ± 0.03	0.11 ± 0.02
0.05	186	0.68 ± 0.21	0.49 ± 0.05	0.04 ± 0.01	0.03 ± 0.01	0.00 ± 0.00
0.1	309	0.51 ± 0.03	0.14 ± 0.03	0.02 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
0.2	460	0.26 ± 0.04	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

MATERIALS AND METHODS

Bacterial strains and inoculum preparation. *Salmonella enterica* serovar Thompson strain RM1987 (cilantro outbreak isolate, courtesy of Dr. M. Brandl, U.S. Department of Agriculture, Agricultural Research Service, Beltsville Agricultural Research Center [USDA-ARS-BARC]), serovar Newport strain F3307 (mango isolate), serovar Poona FS3060 (cantaloupe outbreak isolate, courtesy of J. Karns, USDA-ARS-BARC) and serovar Typhimurium FS3034 (lettuce isolate *(19)*); *E. coli* O157:H7 strains 4052, 4062 (lettuce isolate, donated by M. Donnenberg), 4075 (bagged vegetable outbreak isolate, donated by R. Mandrell, USDA-ARS), and 4156 (spinach outbreak isolate, donated by M. Mammel, U.S Food and Drug Administration); and non-O157 STEC strains O45:H2, O26:H11, O103:H2, and O111:NM were used in this study. Prior to the experiments, stock cultures were streaked on xylose lysine Tergitol 4 (XLT4; BD, Franklin Lakes, NJ) agar (*Salmonella* strains) or sorbitol MacConkey (BD) (*E. coli* O157:H7 and non-O157 STEC strains) and incubated at 37°C for 24 h. A single colony from each strain was grown in tryptic soy broth (TSB; BD) at 37°C for 24 h and washed in phosphate-buffered saline (PBS; BD). Each strain was used individually or combined as a four-strain cocktail (separately for *E. coli* O157:H7, non-O157 STEC, and *Salmonella*) for inoculation.

Preparation of chlorine solutions and water-quality measurement. Chlorine solutions with ~2.0 mg/liter FC were prepared by diluting 6% NaOCl (Clorox, Oakland, CA) in distilled water (22°C) and adjusting to pH 6.5 ± 0.1 with citric acid. This was followed by serial twofold dilutions to obtain solutions with low FC concentrations. The pH of the diluted solutions was also maintained with citric acid at 6.5 ± 0.1. Distilled water without any chlorine (0.0 mg/liter FC) was used as a control. All chlorine concentrations in the solutions were measured with a chlorine photometer (CP-15, HF Scientific, Inc., Ft. Myers, FL).

Preparation of lettuce and tomato extracts used as organic load simulators. Lettuce (*Lactuca sativa* L.) and tomato (*Solanum lycopersicum* Mill.) extracts were prepared from fresh iceberg lettuce, and fresh, mature green tomatoes by using a juice maker (Breville, model BJE200XL, Juice Fountain, Shanghai, China). Lettuce and tomato juices were filtered through two layers of cheesecloth and then centrifuged at 4,629 × *g* (10 min at 4°C) to remove coarse particles. Supernatants were further filtered through a 0.45-μm membrane and then diluted in sterile PBS to make appropriate lettuce extract (LE) or tomato extract (TE) solutions to be used as organic load simulators. The extracts were added to the chlorine solutions immediately before introduction of the pathogens. Chemical oxygen demand (COD) of the solutions was determined with a reactor digestion method (COD2 Mercury-Free COD Reagent, HACH Co., Loveland, CO) *(15)*.

Inactivation of pathogens in chlorine solutions without organic loads. Four strains each of *Salmonella, E. coli* O157: H7, and non-O157 STEC were used in this study. Aliquots of 9.8-ml chlorinated wash solutions (FC, 0.0 to 2.0 mg/liter) were added to 12-strip deep-well microplates. For each bacterial strain, 0.2 ml of diluted culture was added to individual wells and mixed immediately with a multichannel pipette. After exposure for 5, 10, 15, 30, 45, 60, 90, or 120 s, 1.0 ml of liquid from each well was immediately transferred to a prepared 96-well microplate containing 1 ml of 2× TSB plus 0.1% sodium pyruvate and dechlorinating reagent (active ingredient, sodium thiosulfate; Hach) to stop the reaction. The survival of bacterial cells after the treatments were quantitatively determined by plating 0.1 ml of TSB solution onto XLT4 agar plates (*Salmonella* strains) or sorbitol MacConkey agar (*E. coli* O157:H7 or non-O157 STEC strains), and incubated at 37°C for 24 h.

Effect of organic loads on chlorine concentrations and pathogen inactivation. The effect of organic load on chlorine concentration was assessed by adding chlorine solution to varying concentrations of LE or TE. Chlorine water (1 ml, FC of 77 to 79 mg/liter) was added to a 12-well microplate containing 9 ml of LE (0 to 1.25%) or TE (0 to 1.25%) to reach the initial (before reacting LE or TE with the chlorine) FC concentration of 7.7 to 7.9 mg/liter in each well. After 2 min of reaction, residual (after reacting LE or TE with chlorine) FC concentration, turbidity, and COD of the solutions were determined with a chlorine meter, a turbidity meter (AQUAfast, Thermo Orion Research, Inc., Beverly City, MA), and the reactor digestion method *(15)*.

TABLE 2. *Residual FC concentration as impacted by the amount of TE–organic load and the initial FC concentration*

		Residual FC concn (mg/liter) with initial concn (mg/liter) of:				
TE (%)	COD (mg/liter)	2.15 ± 0.11	1.11 ± 0.07	0.58 ± 0.09	0.27 ± 0.03	0.13 ± 0.01
0	123	1.88 ±0.07	0.89 ± 0.17	0.49 ± 0.08	0.23 ± 0.03	0.10 ± 0.03
0.05	200	0.59 ± 0.06	0.48 ± 0.07	0.03 ± 0.01	0.03 ± 0.01	0.00 ± 0.00
0.1	338	0.50 ± 0.01	0.08 ± 0.02	0.02 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
0.2	574	0.08 ± 0.02	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

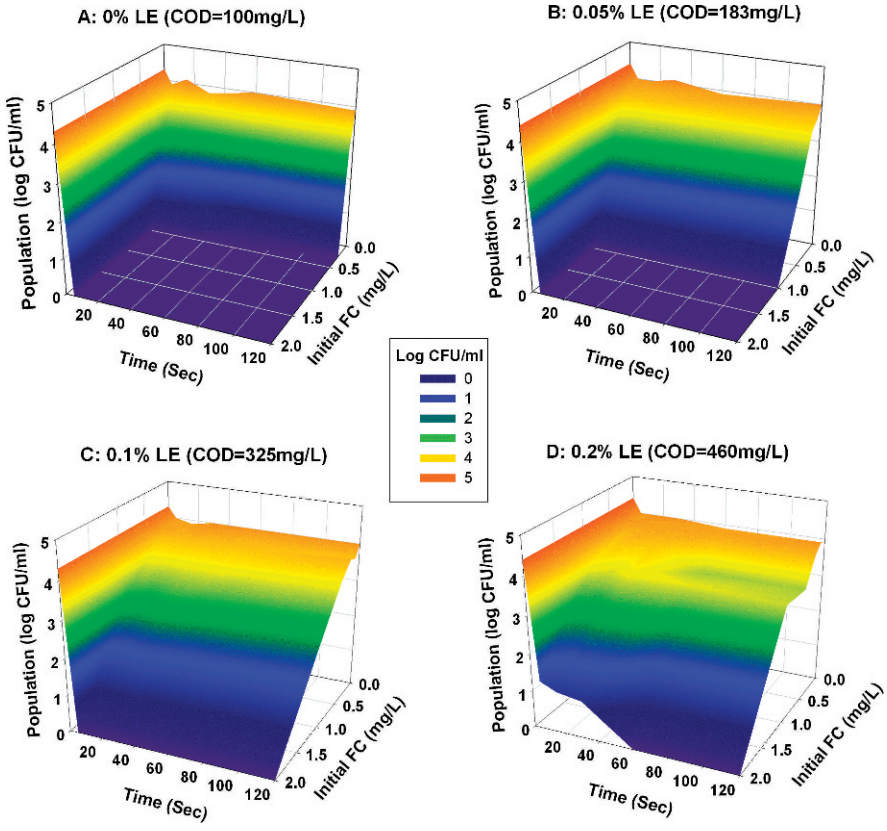

FIGURE 5. *The effects of organic load (LE), initial FC concentration, and exposure time on the survival of* Escherichia coli *O157:H7 cocktails.*

In order to assess the effect of chlorine on pathogen inactivation in the presence of organic matter, four-strain cocktails of *Salmonella, E. coli* O157: H7, and non-O157 STEC (0.2 ml) were prepared in PBS containing 0, 2.5, 5, and 10% LE (for *E. coli* O157:H7 or non-O157 STEC) or TE (for *Salmonella*). Each pathogen cocktail extract mixture was added to 9.8 ml of chlorinated water (FC, ~0.0 to 2.0 mg/liter) in a 12-well microplate and mixed immediately. After 5, 15, 30, 60, or 120 s of exposure, 1.0 ml of liquid from each of the 12 microplate wells was used for pathogen enumeration, as described above. The initial

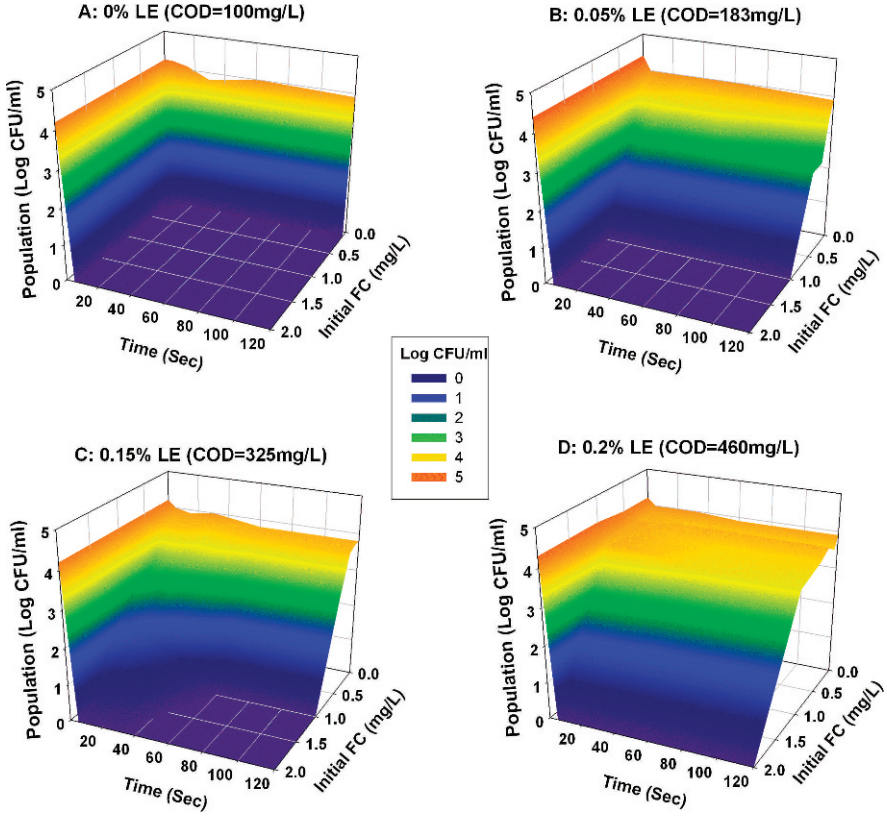

FIGURE 6. *The effects of organic load (LE), initial FC concentration, and exposure time on the survival of non-O157 STEC cocktails.*

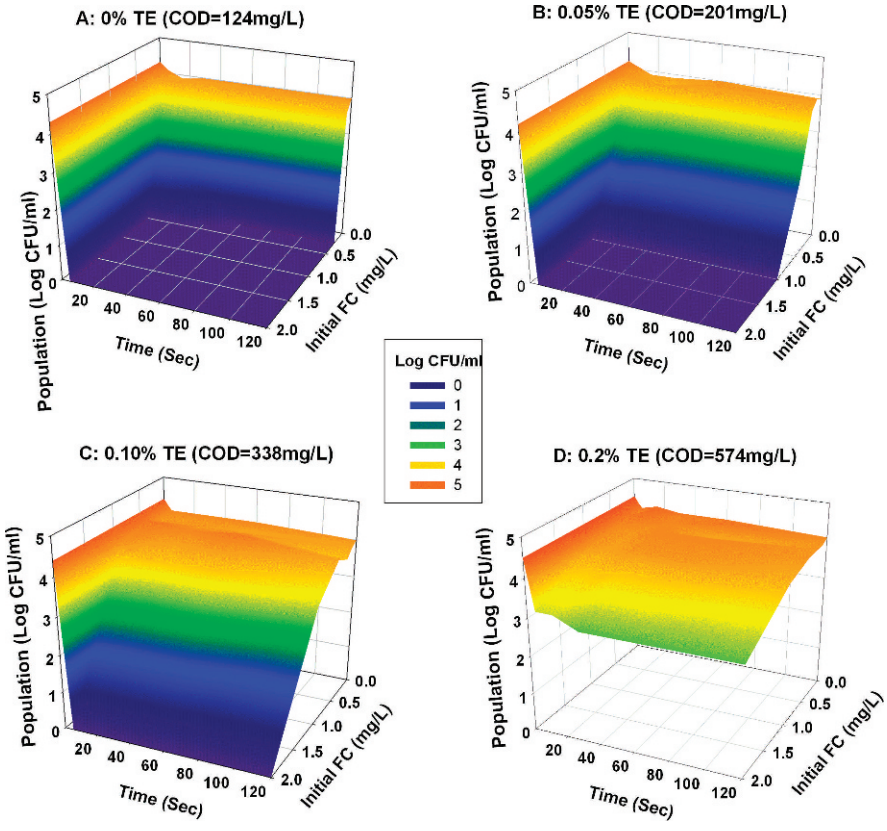

FIGURE 7. *The effects of organic load (TE), initial FC concentration and exposure time on the survival of* Salmonella.

and residual FC concentrations, and COD in solutions in the 12-well microplate were determined with the *N*,*N*-diethyl-*p*-phenylenediamine and reactor digestion method, as described above.

Experimental design and statistical analysis. The experiments were conducted with a factorial design with three to four replications. Microbiological data (survivors or reductions, converted to log CFU per milliliter) were analyzed as a three-factor model by using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC), with independent variables including bacterial strain, FC concentration, and contact time, or FC concentration, organic load, and contact time for cocktail inocula. FC data were also analyzed as a three-factor model with the three independent variables including initial chlorine concentration, COD, and extract type (lettuce or tomato). Water-quality measurements for turbidity, COD, and residual FC were also analyzed as a function of extract concentration and extract type. For all studies, assumptions of normality and variance homogeneity of the model were checked. Means and standard deviations were calculated for all data, and mean significant differences ($\alpha = 0.05$) among interactions were separated with the least significant difference procedure.

RESULTS AND DISCUSSION

FC concentration and exposure time on pathogen inactivation. Pathogen inactivation is a function of FC concentration ($P < 0.0001$) and exposure time ($P < 0.0001$) and varied significantly ($P < 0.0001$) for different bacterial strains (Figs. 1 through 3). When FC concentration equaled or exceeded 0.5 mg/liter and exposure time equaled or exceeded 30 s, all of the tested strains, with the exception of *Salmonella* Newport, were reduced to a level below the detection limit (1.0 log CFU/ml) and achieved a reduction of at least 4.5 log CFU/ml. In fact, at this chlorine level, all pathogens were reduced below the detection level (4.5 log CFU/ml reduction) within 5 s, with the exception of *Salmonella* Newport strain 3558 and *Salmonella* Typhimurium strain 3034. For most organisms, a longer exposure time of 30 to 60 s was required to achieve maximal pathogen inactivation (4.5-log CFU/ml reduction) at lower FC concentrations. When chlorine concentrations were ≤0.14 mg/liter, only *E. coli* O103:H2 and *E. coli* O111:NM were reduced to below detection levels (4.5-log CFU/ml reduction), even with 120-s exposure time. These results suggest that a minimum of 0.5 mg/liter FC and a contact time of 30 s are necessary to inactivate most pathogens in suspension to below detection levels (4.5-log CFU/ml reduction). Recent studies from Yang et al. *(26)* and Luo et al. *(17)* also reported that no *E. coli* O157:H7 survival was detected in solutions with FC ≥0.4 mg/liter after 30-s exposure, and a >4.0-log MPN/ml reduction of four *E. coli* O157:H7 strains after exposure to 0.125 mg/liter FC for 30 s, respectively. Similar results on *E. coli* O157:H7 inactivation by chlorinated solutions were reported by others *(9, 20, 27, 28)*.

Among the tested pathogens, *Salmonella* strains as a group were more resistant than *E. coli* strains to low levels of FC in solution, and non-O157 STEC strains were generally more susceptible than *E. coli* O157:H7 strains to chlorine solution. An FC concentration of 0.25 mg/liter and exposure time of 120 s reduced populations of *Salmonella* serovars Newport USDA 3558, Typhimurium strain 3034, and Thompson strain 1987 by only 2.1 to 3.7 log CFU/liter, while a 30-s exposure to the same FC concentration completely inactivated *E. coli* O103:H2, O111:NM, and O26:H11 strains. Non-O157 STEC strains are frequently

implicated in outbreaks associated with meat consumption *(8),* but they have also been responsible for some outbreaks related to fresh produce, including outbreaks attributed to contaminated salad from a salad bar in Texas (1999) and iceberg lettuce served in a fast food restaurant in Utah (2006) *(18).* Non-O157 STEC strains are a class of emerging pathogens *(18)* that have not (yet) been the subject of many studies. Currently, no information is available regarding the chlorine concentration and contact time needed to inactivate these pathogens. The results obtained in this study suggest conditions that inactivate *Salmonella* and *E. coli* O157:H7 are adequate to inactivate non-O157 STECs.

Impact of organic load on pathogen inactivation by chlorine. Chlorine is widely used by the fresh and fresh-cut produce industry to prevent pathogen survival and cross-contamination during produce washing. Although it is well known that the commercial produce wash solutions contain large amounts of organic materials, and that chlorine can react with organic matter rapidly, no information is available on the dynamic effect of exudates from fresh-cut produce on the changes in wash-water quality and their consequential effect on residual FC concentration and pathogen inactivation. As shown in Figure 4, increasing concentrations of LE or TE introduced to chlorinated water resulted in rapid increases in solution organic load, as indicated by the rapid increase in COD and turbidity values. Increasing LE from 0 to 1.0% in wash water caused the turbidity and COD to increase from 0.03 to 5.26 nephelometric turbidity units (NTU) and from 120 to 880 mg/liter, respectively. Concurrently, the FC decreased from the initial 8.0 to 0.09 mg/liter. When using TE as the organic load simulator, the increase in TE from 0 to 1.0% resulted in the increase in COD to 780 mg/liter and turbidity to 6 NTU (Fig. 4B). This trend agrees well with our survey on Florida tomato packing house dump-tank water quality *(21)* and a study on washing shredded lettuce at a commercial pilot plant *(16).* In these studies, we found that the commercial tomato packing house dump-tank water COD increased to 732 mg/liter, and the COD and turbidity of leafy green wash water increased to 1,400 mg/liter and 24 NTU, respectively.

In order to evaluate the impact of organic load on pathogen inactivation by FC, four-strain cocktails of *Salmonella, E. coli* O157: H7, and non-O157 STEC inocula were chosen to minimize the influence of strain-related differences in sensitivity. A series of wash solutions were prepared with 0, 0.05, 0.1, and 0.2% of LE (Table 1) or TE (Table 2). LE was used for studies related to *E. coli* O157:H7 and non-O157 STEC, and TE was chosen for *Salmonella,* as lettuce and tomatoes are commonly associated with the presence of *E. coli* O157:H7 and non-STEC, and *Salmonella,* respectively. The concentration of organic materials in the chlorine solution significantly affected pathogen reduction. As shown in Figures 5 through 7 and Tables 1 and 2, with the increase in organic load, the initial FC concentrations and exposure time needed to inactivate pathogens significantly increased because of the rapid reaction between chlorine and organic materials and the consequent loss of FC in the solution. In the absence of LE or TE, an initial FC concentration of 0.5 mg/liter or more reduced the *E. coli* O157:H7 in suspension to below detection level, with a brief contact time (Fig. 5A). However, in the presence of 0.1% LE (COD level of 325 mg/liter) (Fig. 5C and Table 1), a significantly higher initial FC concentration and longer contact time are needed to inactivate the *E. coli* O157:H7 to below detection.

Non-O157 STEC cells appeared to be more susceptible to chlorine treatment than *E. coli* O157:H7 or *Salmonella* in the presence of 0.1% LE, as was also found in the absence of organic load. In the presence of 0.1% LE, exposure to an initial FC concentration of 1.0 mg/liter for 60 s, or to an initial FC concentration of 2.0 mg/liter for 5 s was sufficient to inactivate non-O157 STEC cells to below detection (Fig. 6C). On the other hand, *Salmonella* showed greater resistance to chlorine than the other pathogens in the presence of TE, as it did in the absence of organic load.

Many studies have been conducted to investigate the effect of chlorine concentration on pathogen reduction. However, most previous laboratory studies only reported the initial FC concentration *(1, 5, 13),* while the final or the residual FC concentration was less adequately examined *(10).* A comparison of the final FC concentrations in the solutions with different organic loads (Tables 1 and 2) revealed that in the presence of varying organic load, the final or the residual FC concentration is the key factor influencing pathogen inactivation. The final FC concentration remaining in the wash solution after satisfying the chlorine demand associated with the organic matter is the true chlorine concentration available for inactivating pathogens.

In general, more than 4.5-log CFU/ml pathogen reduction was found when the residual FC was $\geq$0.48 for an exposure time $\geq$30 s, regardless of COD values. Residual FC $>$1.23 to 1.55 mg/liter inactivated all three groups of bacterial pathogens within a brief contact. However, it is worth noting that this study only evaluated the exposure time $\geq$5 s because of the practical difficulty of performing the experiments with shorter exposure time. As pathogens released from contaminated produce could be reattached to other produce instantaneously and thus be protected from further sanitizer actions, significantly higher chlorine concentration is needed to prevent pathogen cross-contamination during produce wash *(17).*

During the fresh-cut produce wash process, maintaining an effective FC level is extremely important to ensure the sanitizer efficacy for reducing microbial pathogens and to prevent potential cross-contamination. However, maintaining a constant FC concentration during commercial fresh-cut processing is a serious technical challenge due to the rapid accumulation of organic matter in the wash water. The consumption of FC by organic materials in wash water accelerates exponentially as organic load increases *(16).* Continued replenishment of chlorine in combination with the simultaneous accumulation of by-products can deplete the wash solution of the dissolving power, resulting in the concomitant release of noxious Cl_2 fume. Improved tools and practices are needed for the fresh-cut produce industry to reliably manage FC to accommodate frequent surges in

organic load and their impact on wash-water quality in commercial-scale processing. Understanding the dynamic interactions between organic load and FC concentration is critical to developing practical sanitization strategies for maintaining safety of fresh-cut produce.

In summary, *E. coli* O157:H7, non-O157 STEC, and *Salmonella* cells are sensitive to FC in wash water. Generally, an over 4.5-log CFU/ml reduction in pathogen was found in the solutions when the residual FC concentration was >0.5 mg/liter for longer than 30 s. However, chlorine loses efficacy quickly as a result of its rapid reaction with organic matter, leading to the potential for pathogen survival in wash water. Therefore, maintaining an adequate level of FC is critical for pathogen inactivation in wash water and for prevention of cross-contamination.

ACKNOWLEDGMENT

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